THE BACTERICIDAL SUBSTANCE IN LEUKOCYTES.*

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WYSSOKOWITCH[1] was the first to call attention to the bactericidal power of the blood of living animals, and to show that non-pathogenic bacteria can be recovered from the organs of the body after their disappearance from the blood. Von Fodor[2] first showed that *B. anthracis* was destroyed by freshly drawn blood. Shortly afterward Nuttall[3] proved that while rabbit's-blood serum destroyed *B. anthracis*, this destroying power was lost on the application of heat or upon standing. Nissen[4] working with rabbit's blood pointed out that only certain organisms were destroyed by it, and that others were only retarded in growth or were entirely unaffected. Thus, *Sp. choleras*, *B. anthracis*, *B. typhosus*, and the pneumococcus were killed, whereas *Staph. aureus* and *albus*, and *Strept. pyogenes* were only retarded in growth. He further showed that the blood serum was capable of destroying a limited number of organisms only, and that the time required for this destruction varied with the different organisms. Thus, he found that *Sp. cholerae* required 10 to 40 minutes, *B. typhosus*, about two hours for death by blood serum.

Since these investigations were published a vast amount of research has been directed toward elucidating the intimate relations existing between the properties of the blood and the state of natural susceptibility or of resistance to infective processes. Behring,[5] Buchner,[6] Ogata[7], and many others have obtained remarkable results on the subject, and have given credence to the so-called Buchner's alexin or humoral theory. Metchnikoff and his school,[8] on the contrary, in the face of opposition lasting many years, have offered convincing proofs of the importance of phagocytosis in the protection of the animal body against bacterial invasion. The main theses of the

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[1] *Ztschr. f. Hyg.*, 1886, 1, p. 1.
[2] *Deut. med. Wchnschr.*, 1886, 12, p. 617.
[3] *Ztschr. f. Hyg.*, 1888, 4, p. 353.
[4] *Ibid.*, 1889, 6, p. 487.
[5] *Ibid.*, 1890, 8, p. 412.
[6] *Centralbl f. Bakt.*, 1889–1890, 5, p. 817; 6, pp. 1, 561; 8, p. 65.
[7] *Ibid.*, 1891, 9, p. 597.
[8] *Immunity in Infective Diseases*, tr., Cambridge, 1905.

Metchnikoff theory are now almost universally accepted, but the exact mechanism of the processes involved is still the subject of keen controversy. For a long time an uncompromising opposition divided the workers in the field of immunity into two parties. Of late, however, a growing tendency is manifesting itself toward a fusion of the rival schools of the humoralists and of the supporters of the phagocytic theory. It is not surprising, however, that on many points, and these not the least important, a bewildering discordance of view still exists. One of the most interesting and vital of these issues relates to the rôle of the leukocytes as the originators of the bactericidal substances in the defensive mechanism of the animal organism. In view of this controversy the following experiments made by the writer may be of interest.

METHOD.

The leukocytes used were those of normal rabbits. To obtain polynuclear leukocytes an intrapleural injection of 5 to 10 c.c. of plant-casein emulsion (in 0.85 per cent NaCl solution) was made, and 24 hours later the rabbit was bled to death. The pleural cavities contained a copious turbid exudate. This was pipetted into normal-salt solution with a trace of 1 per cent sodium-citrate solution, was centrifugalized, and then washed in normal-salt solution two or three times. It was then disintegrated in ice-salt mixture for one to two hours, and mixed with an equal bulk of normal-salt solution.

For mononuclear leukocytes an intraperitoneal injection of 5 to 10 c.c. of freshly washed guinea-pig erythrocytes suspended in normal-salt solution containing a trace of sodium citrate was made. From 24 to 48 hours later the rabbit was bled to death and the emulsion treated as above.

The bacteria used were young 18 to 24-hour agar colonies emulsified in broth. The suspension contained 0.05 mg. of bacteria to 0.5 c.c. broth.

In all experiments 0.5 c.c. each of leukocytic and bacterial suspension were mixed, kept at 37° C. 30 minutes, 3 hours, 6 hours, and 24 hours, and then plated in agar.

Inactive suspensions were obtained by heating at 55°–60° C. for 30 minutes.

The controls used were the supernatant cell-free fluid of the exudate, inactivated supernatant cell-free fluid of exudate, fresh rabbit serum, inactivated rabbit serum, and leukocytes disintegrated as above.

Organ extracts were also used, portions of the spleen, liver, bone-marrow of a healthy rabbit being excised immediately after death. These were cut in small pieces and broken up in normal-salt solution by means of a glass rod. The blood was removed so far as possible by repeated washings in fresh saline. The tissue was finally disintegrated in ice-salt mixture for one to two hours as was the leukocytic exudate.

I. POLYNUCLEAR LEUKOCYTES.

Experiment 1.—A 2,500-gm. rabbit was injected with casein 24 hours previous to bleeding. Polynuclear leukocytes, 85 per cent in the film stained by Giemsa's method. The results of action upon *B. typhosus* are seen in the following table:

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Polynuclear cells	+*B. typhosus*	∞	++++	+	o
" " inactivated	+ "	∞	∞	∞	∞
Supernatant fluid	+ "	∞	++	o	o
" " inactivated	+ "	++++	∞	∞	∞
Normal rabbit serum	+ "	++++	++++	∞	o
" " " inactivated	+ "	++++	∞	o	∞
N r. s.+Polynuclear cells	+ "	++++	∞	∞	∞
Polynuclear cells		o	o	o	o
B. typhosus		+++++	∞	∞	∞

∞=Countless; +++++=Very many; ++++=Many; +++=Thousands; ++=Hundreds; +=Few; o=No colony

Experiment 2.—A 2,750-gm. rabbit was injected as before with casein 48 hours previous to bleeding. Polynuclear cells in the film stained by Giemsa's method, 90 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Polynuclear cells	+*B. typhosus*	+++++	+++++	∞	∞
" " inactivated	+ "	+++++	∞	∞	∞
Supernatant fluid	+ "	+++++	+++	++	+
" " inactivated	+ "	+++++	∞	∞	∞
Normal rabbit serum	+ "	+++	o	o	o
" " " inactivated	+ "	+++++	+++++	∞	∞
N. r. s.+Polynuclear cells	+ "	+++++	+++	++++	∞
Polynuclear cells		o	o	o	o
B. typhosus		∞	∞	∞	∞

Experiment 3.—A 1,800-gm. rabbit was injected as before with casein 32 hours previous to bleeding. Polynuclear cells in the film stained by Giemsa's method, 80 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Polynuclear cells	+*Sp. cholerae*	+++	++	o	o
" " inactivated	+ "	+++	+++	∞	∞
Supernatant fluid	+ "	+++	++	++	o
" " inactivated	+ "	+++++	∞	∞	∞
Normal rabbit serum	+ "	+++	++	o	o
" " " inactivated	+ "	+++	+++++	∞	∞
N. r. s.+Polynuclear cells	+ "	+++	++	+++++	∞
Polynuclear cells		o	o	o	o
Sp. cholerae		+++	+++++	∞	∞

The experiments on *B. typhosus* and *Sp. cholerae* show that polynuclear leukocytes have bactericidal power. This agrees with Petri's results (1904) as in his experiments with two rabbits the polynuclear cells killed *B. typhosus*.

Experiment 4.—A 1,800-gm. rabbit was injected as before with casein 48 hours previous to bleeding. Polynuclear cells in the film stained by Giemsa's method, 85 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Polynuclear cells	+*B. dysenteriae*	+++++	+++++	∞	∞
" " inactivated	+ "	+++++	∞	∞	∞
Supernatant fluid	+ "	+++++	++++	++	+
" " inactivated	+ "	+++	+++++	∞	∞
Normal rabbit serum	+ "	+++++	++	o	o
" " " inactivated	+ "	+++++	+++++	∞	∞
N. r. s.+Polynuclear cells	+ "	+++	++++	+++++	∞
Polynuclear cells		o	o	o	o
B. dysenteriae		+++++	∞	∞	∞

Experiment 5.—A 1,800-gm. rabbit was injected as before with casein 18 hours previous to bleeding. Polynuclear cells counted, 80 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Polynuclear cells	+*B. dysenteriae*	+++++	∞	∞	∞
" " inactivated	+ "	+++++	∞	∞	∞
Supernatant fluid	+ "	+++++	+++++	+++	++
" " inactivated	+ "	+++++	+++++	∞	∞
Normal rabbit serum	+ "	++++	+++	++	o
" " " inactivated	+ "	+++	+++++	∞	∞
N. r. s.+Polynuclear cells	+ "	+++++	+++	∞	∞
Polynuclear cells		o	o	o	o
B. dysenteriae		+++++	∞	∞	∞

Experiment 6.—A 2,800-gm. rabbit was injected as before with casein 48 hours previous to bleeding. Polynuclear cells counted, 90 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Polynuclear cells	+*B. coli*	+++++	+++++	∞	∞
" " inactivated	+ "	+++++	∞	∞	∞
Supernatant fluid	+ "	+++++	+++++	++++	∞
" " inactivated	+ "	+++++	∞	∞	∞
Normal rabbit serum	+ "	+++++	++++	o	∞ o
" " " inactivated	+ "	+++++	∞	∞	∞
N. r. s.+Polynuclear cells	+ "	+++++	+++++	∞	∞
Polynuclear cells		o	o	o	∞ o
B. coli		+++++	∞	∞	∞

II. MONONUCLEAR LEUKOCYTES.

Experiment 7.—A 2,800-gm. rabbit was injected as before with guinea-pig erythrocytes 32 hours previous to bleeding. Mononuclear cells counted, 50 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Mononuclear cells	+*B. typhosus*	+++++	∞	∞	∞
" " inactivated	+ "	+++++	∞	∞	∞
Supernatant fluid	+ "	+++++	++++	++++	o
" " inactivated	+ "	+++++	∞	∞	∞
Normal rabbit serum	+ "	++++	++++	++	∞ o
" " " inactivated	+ "	+++++	∞	∞	∞
N. r. s.+Mononuclear cells	+ "	+++++	∞	∞	∞
Mononuclear cells		o	o	o	o
B. typhosus		+++++	∞	∞	∞

Experiment 8.—A 2,800-gm. rabbit was injected as before with guinea-pig erythrocytes 48 hours previous to bleeding. Mononuclear leucocytes counted, 40 per cent.

		30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Mononuclear cells	+*Sp. cholerae*	++++	+++++	∞	∞
" " inactivated	+ "	+++++	∞	∞	∞
Supernatant fluid	+ "	+++++	+++++	++++	++
" " inactivated	+ "	+++++	∞	∞	∞
Normal rabbit serum	+ "	++++	++	+	o
" " " inactivated	+ "	++++	+++++	∞	∞
N. r. s.+Mononuclear cells	+ "	++++	++++	∞	∞
Mononuclear cells		o	o	o	o
Sp. cholerae		++++	∞	∞	∞

Experiment 9.—A 2,300-gm. rabbit was injected as before with guinea-pig erythrocytes 24 hours previous to bleeding. Mononuclear cells counted, 50 per cent.

			30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Mononuclear cells	..	*B. dysenteriae*	+++++	∞	∞	∞
" " inactivated	+	"	+++++	∞	∞	∞
Supernatant fluid	+	"	+++++	+++	+++	o
" " inactivated	+	"	+++++	+++++	∞	∞
Normal rabbit serum	+	"	+++++	+++	+	o
" " " inactivated	+	"	+++++	+++++	∞	∞
N. r. s.+Mononuclear cells	+	"	+++++	+++++	∞	∞
Mononuclear cells			o	o	o	o
B. dysenteriae			+++++	∞	∞	∞

Experiment 10.—A 1,950-gm. rabbit was injected as before with guinea-pig erythrocytes 24 hours previous to bleeding. Mononuclear cells counted, 45 per cent.

			30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Mononuclear cells	+	*B. coli*	+++++	+++++	∞	∞
" " inactivated	+	"	+++++	∞	∞	∞
Supernatant fluid	+	"	+++++	+++++	+++	++
" " inactivated	+	"	+++++	∞	∞	∞
Normal rabbit serum	+	"	+++++	+++	++	o
" " " inactivated	+	"	+++++	+++++	∞	∞
N. r. s.+Mononuclear cells	+	"	+++	+++	+++++	∞
Mononuclear cells			o	o	o	o
B. coli			+++++	∞	∞	∞

III. ORGAN EXTRACTS.

			30 Min.	3 Hrs.	6 Hrs.	24 Hrs.
Spleen extract	+	*B. typhosus*	+++++	∞	∞	∞
Liver "	+	"	+++++	+++++	∞	∞
Marrow "	+	"	+++++	+++++	∞	∞
Spleen "	+	*Sp. cholerae*	+++	+++++	∞	∞
Liver "	+	"	+++++	+++++	∞	∞
Marrow "	+	"	+++	∞	∞	∞
Spleen "	+	*B. dysenteriae*	+++++	∞	∞	∞
Liver "	+	"	+++++	∞	∞	∞
Marrow "	+	"	+++	+++++	∞	∞
Spleen "	+	*B. coli*	+++++	+++++	∞	∞
Liver "	+	"	+++++	∞	∞	∞
Marrow "	+	"	+++++	∞	∞	∞

CONCLUSIONS.

The following general conclusions may be drawn from the experiments I have described:

1. No bactericidal substance for *B. typhosus*, *Sp. cholerae*, *B. dysenteriae*, and *B. coli* ordinarily exists in the polynuclear leukocytes obtained from the normal adult rabbit; however, one experiment on *B. typhosus* and one on *Sp. cholerae* showed some bactericidal effect for them.

2. No bactericidal substance for *B. typhosus*, *Sp. cholerae*, *B.*

dysenteriae, and *B. coli* exists in the mononuclear leukocytes obtained from the normal adult rabbit.

3. No bactericidal substance for *B. typhosus*, *Sp. cholerae*, *B. dysenteriae*, and *B. coli* exists in the intracellular products of the spleen, liver, and bone-marrow of the normal rabbit.

4. Normal rabbit serum and supernatant fluid are invariably powerful in their bactericidal effect on *B. typhosus*, *Sp. cholerae*, and *B. dysenteriae*, but when heated at 55° C. to 60° C. for 30 min. the esrum fails in bactericidal action.

As previously, I have applied a disintegrating method to get bac tericidal substance in the body of leukocytes, but the result did not show distinctly any substance similar to "alexin" or "complement;" therefore I question whether the leukocytes have an endolysin as the bactericidal substance. Concerning this I also have doubt as to the bactericidal substance in the leukocytes.

The pleasant duty is incumbent upon me, in conclusion, of recording my sense of indebtedness to Prof. S. Kitasato for his kind suggestion; and I must also thank Prof. K. Shiga for similar services.